

## T4 Endonuclease VII

### IMPORTANCE OF A HISTIDINE-ASPARTATE CLUSTER WITHIN THE ZINC-BINDING DOMAIN\*

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**The DNA junction-resolving enzyme endonuclease VII of bacteriophage T4 contains a zinc-binding region toward the N-terminal end of the primary sequence. In the center of this 39-amino acid section (between residues 38 and 44) lies the sequence HLDHDHE, termed the His-acid cluster. Closely related sequences are found in three other proteins that have similar zinc-binding motifs. We have analyzed the function of these residues by a site-directed mutagenesis approach, modifying single amino acids and studying the properties of the resulting N-terminal protein A fusions. No sequence changes within the His-acid cluster led to a change in zinc content of the protein, indicating that these residues are not involved in the coordination of zinc. We found that the N-terminal aspartate residue (Asp-40) and the two histidine residues (His-41 and His-43) within the cluster are essential for junction-cleavage activity of the proteins. However, all sequence variations within this region generate proteins that retain their ability to bind to four-way DNA junctions (with minor changes in binding affinity in some cases) and to distort their global structure in the same manner as active enzymes. We conclude that the process of cleavage can be uncoupled from those of binding to and distortion of the junction. It is probable that some amino acid side chains of the His-acid cluster participate in the phosphodiester cleavage mechanism of endonuclease VII. The essential aspartate residue might be required for coordination of catalytic metal ions.**

The DNA junction-resolving enzymes are a class of nucleases that recognize the structure of branched DNA molecules. Such enzymes are important in DNA recombination and repair for the processing of four-way DNA junctions created as intermediates (1–10). Junction-selective nucleases have been isolated from bacteriophage-infected eubacteria (11, 12), *Escherichia coli* (13–16), yeast (17, 18), and mammalian cells (19, 20) and their viruses (21) and are very probably ubiquitous cellular enzymes.

These proteins are fundamentally structure-selective. For example, the complexes formed between T7 endonuclease I, T4 endonuclease VII, or yeast CCE1, and four-way DNA junction are not displaced by a 1000-fold excess of duplex DNA of the same sequence (22–24). Although RuvC of *E. coli* and yeast CCE1 exhibit significant sequence specificity, this is manifested at the level of the cleavage reaction, and these enzymes

bind to four-way junctions of any sequence (24, 25). The existence of mutants of T7 endonuclease I and T4 endonuclease VII that bind normally to DNA junctions but are defective in cleavage suggests that binding and catalysis are separable events. While the binding of resolving enzymes is selective for the structure of DNA junctions, the act of binding in general also distorts the global configuration of helical arms (22, 26, 27).

Endonuclease VII of T4 is required during late infection in order to resolve DNA branch points prior to packaging of the DNA into phage heads (11, 28). Examination of the primary sequence of endonuclease VII (29) suggests the existence of three sections that might form modules within the overall protein structure. There is a region at the C terminus that is 48% identical to a sequence within the pyrimidine dimer glycosylase and nuclease T4 endonuclease V. The structure of this repair enzyme is known (30), and the region of similarity comprises a helix and an extended section. Replacement of the region of endonuclease VII with the corresponding sequence of endonuclease V resulted in a chimeric protein that retained its specificity for the precise cleavage of four-way DNA junctions (23). In the center of the endonuclease VII sequence is a section with some similarity to a region of the functionally related resolving enzyme T7 endonuclease I. We have previously found that in selection of non-functional mutants of T7 endonuclease I, all such mutants map within this region of the protein (22), suggesting that it may comprise a significant part of the active site for DNA cleavage. We have shown that a mutation within the corresponding part of the primary sequence of T4 endonuclease VII results in a catalytically inactive protein (27).

The N-terminal section of endonuclease VII contains a 40-amino acid region bounded by two Cys-X-X-Cys motifs that binds an atom of zinc (23). This region is 42% identical to a section found in a protein (gp59) of unknown function encoded by mycobacteriophage L5 (31). In addition, an open reading frame identified downstream of the *secF* gene of *E. coli* and *Salmonella typhimurium* (32) encodes a 109-amino acid protein of unknown function that includes a region that is 43% identical with this section, which is also bounded by Cys-X-X-Cys motifs. The four sequences are collected together in Fig. 1. The comparison reveals a number of conserved features, including arginine (position 28 in endonuclease VII), asparagine (position 31), and glycine (position 51). In addition, there is a conserved four-residue sequence Asp-His-Asp-His beginning with aspartate 40 in endonuclease VII. Indeed, this cluster of histidine and acidic residues can be extended in endonuclease VII, beginning with histidine 38, to read HLDHDHE. Acidic residues are frequently involved in the catalytic sites of nucleases (33–36), where they coordinate metal ions that participate in the chemistry of phosphodiester bond cleavage, and we were curious to learn whether any or all of these residues might be involved in catalysis. We have therefore made point mutants of endonuclease VII in which amino acids within the cluster of

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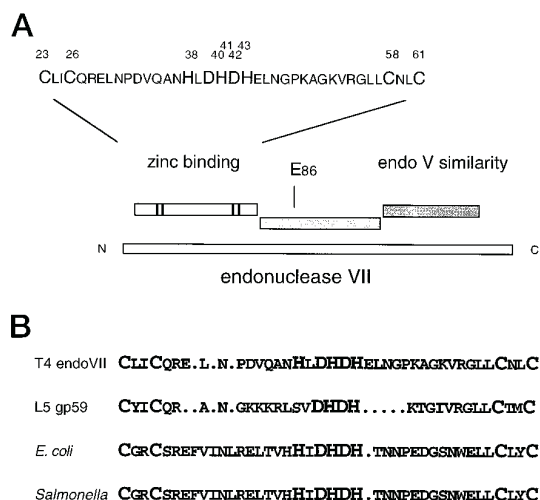


FIG. 1. The potential domain structure of T4 endonuclease VII. A, schematic map of the regions of endonuclease VII, showing the locations of amino acid residues changed in these studies. The N-terminal section of the protein binds a zinc ion (23) that is coordinated by four cysteine residues, and the sequence of this region is shown. The His-acid cluster at the center of the section is highlighted in *larger type*. The C-terminal section is similar to a region of T4 endonuclease V. The central section has some similarity to a region of T7 endonuclease I, and the catalytically inactivating E86A mutation (27) lies in this region. B, an alignment of sequences of probable zinc-binding domains in T4 endonuclease VII, gp59 of mycobacteriophage L5 (31), and open reading frames identified in the *secD* locus of *E. coli* and *S. typhimurium* (32). The sequences are aligned by their CXXC sequences. Note the presence of the His-acid cluster within each of these sequences.

histidine and acidic side chains (referred to subsequently as the his-acid cluster) have been changed to different residues, and we have examined the ability of the resulting proteins to bind to or cleave four-way DNA junctions.

#### MATERIALS AND METHODS

**Synthesis of Oligonucleotides**—Oligonucleotides were synthesized using  $\beta$ -cyanoethyl phosphoramidite chemistry (44, 45) implemented on a 394 DNA/RNA synthesizer (Applied Biosystems). Fully deprotected oligonucleotides were purified by gel electrophoresis in 10% polyacrylamide containing 7 M urea, the bands were excised, and DNA was eluted and recovered by ethanol precipitation.

**Construction of Four-way DNA Junctions**—A four-way DNA junction with four arms of 25 bp<sup>1</sup> was generated by the hybridization of four oligonucleotides of 50 nucleotides each, one of which was radioactively 5'-<sup>32</sup>P-labeled. The oligonucleotides were based upon the sequence of junction 3 studied by Duckett *et al.* (39); thus, the b strand had the sequence 5' CCTCGAGGGATCCGTCCTAGCAAGGGGCTGCTACCGGAAGCTTCTCGAGG 3'.

For the comparative gel electrophoretic analysis of the global structure of the complex between junction and endonuclease mutants, six junctions comprising two long arms of 40 bp and two short arms of 15 bp were each generated by hybridization of appropriate oligonucleotides based on the sequence of junction 3 as described above. As an example, the BH junction (where the B and H arms are long) was obtained using the following four oligonucleotides: b strand, 5' CGCAAGCGACAGGAACCTCGAGGGATCCGTCCTAGCAAGGGGCTGCTACCGGAAGCTTCTCGAGGTTCTGTCGCTTGCG 3'; h strand, 5' CGCAAGCGACAGGAACCTCGAGGAAGCTTCCGGTAGCAGCCTGAGCGGTGGTTGAA 3'; r strand, 5' TTCAACCACCGCTCAACTCAACTGCAGTCT 3'; and x strand, 5' AGACTGCAGTTGAGTCCTTGTAGGACGGATCCCTCGAGGTTCTGTCGCTTGCG 3'.

In each case, stoichiometric quantities of three unlabeled and one radioactively 5'-<sup>32</sup>P-labeled strands were annealed by incubation in 50 mM Tris-HCl (pH 7.6), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM spermidine, and 0.1 mM EDTA for 3 min at 85 °C, followed by slow

cooling. Assembled junctions were purified by gel electrophoresis in 5% polyacrylamide (acrylamide/bisacrylamide, 20:1) gels. Bands were excised and DNA was recovered by electroelution. DNA concentrations were measured spectrophotometrically at 260 nm, using an extinction coefficient of  $\epsilon = 6.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \text{ bp}^{-1}$ .

**Enzymes**—Oligonucleotides were radioactively labeled at their 5' termini using [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase (Amersham). Ligation of DNA was performed using T4 DNA ligase (Amersham) under standard conditions (46).

**Cloning of Endonuclease VII to Encode an Oligohistidine Fusion Protein**—A fragment containing part of the synthetic gene encoding endonuclease VII was obtained by digestion of pAT153-SEVII (23) by *Hind*III and *Bam*HI. The sequence lost from the 5' end of the gene was restored by hybridization of two oligonucleotides, giving a DNA fragment containing an *Nco*I site at the 5' end and a *Hind*III site at the 3' end; this also added the coding sequence for 10 histidine residues and a site for proteolytic cleavage by enterokinase. This was ligated into pET-19b (previously digested by *Nco*I and *Bam*HI) to generate the plasmid pETSEVII, which was used to transform the *E. coli* strain HMS174(DE3) pLysS.

**Mutagenesis by Polymerase Chain Reaction**—Mutagenesis of the synthetic endonuclease VII gene was performed by polymerase chain reaction (PCR) using one primer that differed in sequence from the synthetic gene sequence by one or two nucleotides (therefore changing the appropriate codon) and one non-mutated primer. In order to maximize the stability of the hybrid, at least seven nucleotides were placed between the mutagenic mismatch(es) and the ends of the oligonucleotide. In all cases, the mutated primer extended to the nearer restriction site to facilitate the cloning. For example, the sequence of the primer used for changing the histidine 38 to a glutamine was 5' CTCAATC-CAGACGTC<sup>CAAGCTAATCAACTTGACCAC</sup> 3', where the *Aat*II cloning site and mutagenic adenosine base are underlined. The other primer corresponded to a sequence downstream to the targeted area, 5' CCACGTTGCAGCATCTCTGC 3'.

PCR reactions were performed as described by Landt *et al.* (47) using 1 unit of *Taq* DNA polymerase, 1 ng of plasmid DNA, 100 pmol of each primer, 50  $\mu$ M dNTPs in 50 mM Tris-HCl (pH 9), 1 mM MgCl<sub>2</sub>, 0.1% Triton X-100 for 30 cycles (1 min at 93 °C, 1 min at 45 °C, and 45 s at 72 °C). The PCR products were digested with the restriction enzymes *Aat*II and *Afl*III, and the fragment was purified by gel electrophoresis and used to replace the wild-type sequence in pK19SEVII (23).

**DNA Sequencing**—The base sequences of wild-type and mutated genes were obtained by primer extension-dideoxy sequencing (48).

**Preparation of Oligohistidine-Endonuclease VII Fusion Protein from E. coli**—Endonuclease VII as an oligohistidine fusion protein was prepared from 1 liter of *E. coli* strain HMS 174 (DE3) pLysS transformed with pETSEVII. The cells were grown to an  $A_{600}$  of 0.6 and then induced with IPTG to a final concentration of 1 mM for 2 h. Cells were harvested, resuspended in 20 ml of 20 mM Tris-HCl (pH 7), 0.5 M NaCl, 1 mM imidazole, and lysed by sonication. Unbroken cells and cell debris were removed by centrifugation (40,000  $\times g$  for 15 min). The protein was purified by affinity chromatography using a Fractogel EMD chelate column previously charged with nickel chloride. The protein was eluted using a gradient of imidazole from 0 to 500 mM in 20 mM Tris-HCl (pH 7), 0.5 M NaCl. The protein-containing fractions were pooled and dialyzed for 2 h at 4 °C against 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 50% glycerol and were stored at -20 °C.

**Preparation of Protein A-Endonuclease VII Fusion Proteins from E. coli**—*E. coli* strain JM101 transformed with the appropriate protein A-fusion plasmids based on the pK19 system (37) was grown to an  $A_{600}$  of 0.6 and induced with 0.5 mM IPTG for 2 h. Cells were harvested by centrifugation and resuspended in 20 ml of 20 mM MES (pH 6). Cells were lysed by sonication as described above. Two ammonium sulfate precipitation steps were performed. Ammonium sulfate was added to 40% saturation and the precipitate was discarded. Further ammonium sulfate was added to the supernatant to a final concentration of 65% saturation. The pellet was redissolved in 5 ml of a solution of 20 mM MES (pH 6), 1 mM DTT and was applied to an S-Sepharose ion-exchange column. A gradient of NaCl in the same buffer was applied to the column. The peak fractions containing the protein were dialyzed against 20 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 50% glycerol for 2 h at 4 °C. The concentration of the protein was determined by the Bradford method, calibrated against a previous amino acid analysis for endonuclease VII-H41T (23). The purity of the proteins obtained was verified by polyacrylamide gel electrophoresis in the presence of SDS.

**Preparation of Endonuclease VII H38T Without Fusion Polypeptide**—Protein A-endonuclease VII H38T was prepared as described above, but after elution from the S-Sepharose column the pooled fractions were not

<sup>1</sup> The abbreviations used are: bp, base pair(s); PCR, polymerase chain reaction; IPTG, isopropyl-1-thio- $\beta$ -D-galactopyranoside; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; PMPS, *p*-hydroxymercuriphenylsulfonic acid; PR, 4-(2-pyridylazo)resorcinol.

dialyzed in the glycerol-containing solution. The buffer was changed by centrifugation in a Centrplus concentrator (Amicon) to 20 mM sodium phosphate (pH 7.4), and the sample was concentrated to 5 ml. The protein was digested overnight with a ratio of 1:500 (w/w) of protease factor Xa. The digested protein was reapplied to the S-Sepharose column. The released protein A was not retained by the resin, and non-fusion endonuclease VII H38T was eluted using a gradient of NaCl in 20 mM MES (pH 6), 1 mM DTT. The protein-containing fractions were dialyzed in 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 50% glycerol for 2 h at 4 °C.

**Cleavage of Four-way DNA Junctions**—Reactions were performed on ice in 10  $\mu$ l of 112 nM four-way DNA junction 3 individually 5'-<sup>32</sup>P-labeled on either the b, h, r, or x strand and endonuclease VII or mutant protein in 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM DTT, 100  $\mu$ g/ml bovine serum albumin, 10 mM MgCl<sub>2</sub> (cleavage reaction buffer) for 20 min. The reactions were terminated by addition of 10  $\mu$ l of formamide, 50 mM EDTA. The samples were loaded on a 10% polyacrylamide denaturing gel (acrylamide/bisacrylamide, 29:1). After electrophoresis the gels were dried onto Whatman 3MM paper and subjected to autoradiography at -70 °C using Fuji RX x-ray film with Ilford fast tungstate intensifier screens.

**Gel Electrophoretic Retardation Analysis of Protein A-Endonuclease VII Mutants and Measurement of the Apparent Binding Constants**—Varying amounts of each mutant and wild-type protein were incubated with 24.2 nM 5'-<sup>32</sup>P-labeled junction for 10 min at room temperature in 10  $\mu$ l of binding buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM dithiothreitol, and either 1 mM EDTA or 200  $\mu$ M MgCl<sub>2</sub>). After addition of 2  $\mu$ l of 35% (w/v) Ficoll solution, bound and free junctions were separated by electrophoresis in a 6% polyacrylamide gel (acrylamide/bisacrylamide, 20:1) in the presence of either TBE (90 mM Tris borate (pH 8), 1 mM EDTA) or TBM (90 mM Tris borate (pH 8), 200  $\mu$ M MgCl<sub>2</sub>). The electrophoresis was performed at 110 V for 4–6 h. Magnesium salt-containing buffers were continuously recirculated at 1 liter/h. Dried gels were subjected to autoradiography, and the radioactivity present in different bands was quantified as described above. The fraction of DNA bound to protein ( $f_b$ ) was calculated for each protein concentration, and the association constant ( $K_a$ ) was calculated by fitting the data by regression analysis to the equation,

$$f_b = \frac{(1 + K_a P_T + K_a D_T) - \sqrt{(1 + K_a P_T + K_a D_T)^2 - (4D_T K_a^2 P_T)}}{2D_T K_a} \quad (\text{Eq. 1})$$

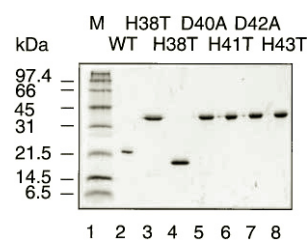
where  $P_T$  is the total protein concentration (calculated as a dimer) and  $D_T$  is the total DNA concentration. The dissociation constant ( $K_D$ ) is the reciprocal of  $K_a$ .

**Comparative Gel Electrophoretic Analysis of the Global Structure of Protein-bound Four-way Junctions**—Protein A fusions of wild-type and mutant endonuclease VII were incubated separately with each of the six forms of 5'-<sup>32</sup>P-labeled junction containing two arms of 40 bp and two of 15 bp (see above) for 10 min at room temperature in 10  $\mu$ l of binding buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM dithiothreitol, and either 1 mM EDTA or 200  $\mu$ M MgCl<sub>2</sub>). After addition of 2  $\mu$ l of 35% (w/v) Ficoll solution, the different species were separated by electrophoresis in a 6% polyacrylamide gel (acrylamide/bisacrylamide, 20:1) in the presence of either TBE or TBM. Electrophoresis was performed at 110 V for 16 h.

**Determination of Zinc Bound to Protein**—Colorimetric assays were performed as described by Giedroc *et al.* (38) as applied to T4 endonuclease VII (23). The proteins were dialyzed overnight in 20 mM Tris-HCl (pH 8), 600 mM NaCl, and 5% glycerol. 4-(2-Pyridylazo)resorcinol (PAR) was added to 800  $\mu$ l of a 5  $\mu$ M protein solution to a final concentration of 0.1 mM. The protein-resorcinol solution was titrated with *p*-hydroxymercuriphenylsulfonic acid (PMPS). The zinc released by PMPS was chelated by the resorcinol, and the titration was followed by measuring the absorbance of Zn(II)PAR<sub>2</sub> at 500 nm (49). The concentration of protein-bound zinc was calculated from a titration of standard ZnCl<sub>2</sub> solutions with resorcinol obtained under the same conditions. Absorption measurements were performed on a Cary 1E UV-visible spectrophotometer using 1-ml polystyrene cuvettes. All buffers were treated with the chelating resin Chelex 100 (Sigma). Protein concentrations required for zinc determination were measured by Bradford assay using a control sample whose concentration was obtained by amino acid analysis on an Applied Biosystems 420H analyzer.

## RESULTS

**Construction and Expression of Endonuclease VII Mutants**—We have constructed a series of point mutants within



**FIG. 2. Gel electrophoresis of endonuclease VII and derived mutant proteins.** The purified proteins were separated by SDS-polyacrylamide gel electrophoresis. *Track 1*, a mixture of proteins to serve as size standards (molecular masses indicated at left, in kDa); *track 2*, wild-type sequence endonuclease VII as N-terminal oligohistidine fusion (calculated mass, 21 kDa); *track 3*, protein A-endonuclease VII H38T (calculated mass, 36 kDa); *track 4*, endonuclease VII H38T released from fusion with protein A by Factor Xa cleavage and purification by chromatography (calculated mass, 18 kDa); *track 5*, protein A-endonuclease VII D40A (calculated mass, 36 kDa); *track 6*, protein A-endonuclease VII H41T (calculated mass, 36 kDa); *track 7*, protein A-endonuclease VII D42A (calculated mass, 36 kDa); *track 8*, protein A-endonuclease VII H43T (calculated mass, 36 kDa).

**TABLE I**  
*Mutations introduced into the His-acid region of endonuclease VII and their properties*

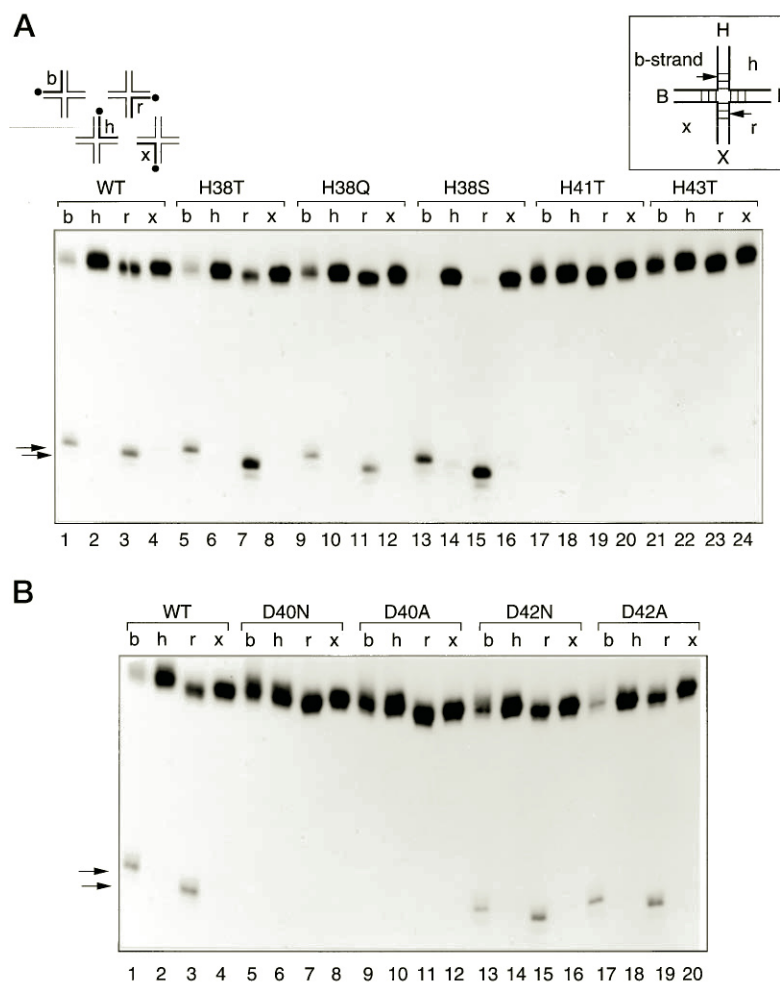
The wild-type sequence protein was studied as an N-terminal oligohistidine fusion, while all the mutant proteins were analyzed as N-terminal protein A fusions. Dissociation constants were calculated by measuring the extent of binding to DNA junctions of the proteins as a function of their concentration, fitting the data as described under "Materials and Methods." These data were all measured in the absence of added magnesium ions. For protein A-endonuclease VII H38T the error is the standard error obtained from three independent experiments; for the other proteins the errors are derived from the fit of individual data points. Zinc stoichiometries were measured using a colorimetric assay. The errors are the random error on the data points in the absorption plateau region, and the full experimental error is probably larger than this.

Mutation	Activity	$K_D$	
		nM	mol/mol
Wild type	Active	38 $\pm$ 16	
H38T	Active, but thermosensitive	45 $\pm$ 2	1.1 $\pm$ 0.08
H38Q	Active, but thermosensitive	25 $\pm$ 16	
H38S	Active, but thermosensitive	5.3 $\pm$ 5	
D40N	Inactive	20 $\pm$ 8	
D40A	Inactive	20 $\pm$ 19	1.02 $\pm$ 0.01
H41T	Inactive	96 $\pm$ 47	0.94 $\pm$ 0.01
D42N	Active	25 $\pm$ 20	
D42A	Active	28 $\pm$ 15	0.81 $\pm$ 0.03
H43T	Very low activity	6 $\pm$ 2	0.99 $\pm$ 0.02

the His-acid cluster of T4 endonuclease VII by means of site-directed mutagenesis of the synthetic gene described previously (23). A section of the gene was replicated by means of the PCR that included one mutagenic primer. The amplified fragment was cleaved with restriction enzymes and ligated into the corresponding location in the synthetic gene. The sequence of the mutated gene was confirmed by DNA sequencing.

We have previously shown that N-terminal fusions of protein A, maltose binding protein, or oligohistidine affect neither the cleavage of nor the binding to four-way DNA junctions. Moreover, the increased molecular mass of such mutants actually confers an experimental advantage for electrophoretic retardation experiments. The mutant genes were therefore cloned as translational fusions with protein A in the plasmid pK19PRA (37) and transformed in *E. coli* JM101. Expression was under the control of the *lac* promoter and was induced by the addition of IPTG. Following ammonium sulfate precipitation, the protein A fusion polypeptides were purified by ion exchange chromatography. The endonuclease VII variants could be released from the protein A fusion by digestion with Factor Xa protease.





**FIG. 3. Comparison of the cleavage of a four-way DNA junction by endonuclease VII and derived mutant proteins.** Four-way DNA junction 3 was prepared radioactively  $5'$ - $^{32}\text{P}$ -labeled individually in the b, h, r, or x arm. These species (112 nM) were each incubated with endonuclease VII or mutant protein on ice, and the products of the reaction analyzed by electrophoresis on a sequencing gel and by autoradiography. Endonuclease VII cleaves junction 3 primarily at single phosphodiester bonds on the b and r strands, shown by the arrows drawn on the schematic of the junction. The wild-type sequence enzyme was generated as an N-terminal oligohistidine fusion, while all the mutant proteins have been studied as N-terminal protein A fusions. **A**, histidine mutants. *Tracks 1–4*, incubation with 180 nM oligohistidine endonuclease VII; *tracks 5–8*, incubation with 60 nM protein A-endonuclease VII H38T; *tracks 9–12*, incubation with 60 nM protein A-endonuclease VII H38Q; *tracks 13–16*, incubation with 60 nM protein A-endonuclease VII H38S; *tracks 17–20*, incubation with 1  $\mu\text{M}$  protein A-endonuclease VII H41T; *tracks 21–24*, incubation with 1  $\mu\text{M}$  protein A-endonuclease VII H43T. *Tracks 1, 5, 9, 13, 17, and 21*, junction 3  $5'$ - $^{32}\text{P}$ -labeled on the b strand; *tracks 2, 6, 10, 14, 18, and 22*, junction 3  $5'$ - $^{32}\text{P}$ -labeled on the h strand; *tracks 3, 7, 11, 15, 19, and 23*, junction 3  $5'$ - $^{32}\text{P}$ -labeled on the r strand; *tracks 4, 8, 12, 16, 20, and 24*, junction 3  $5'$ - $^{32}\text{P}$ -labeled on the x strand. **B**, aspartate mutants. *Tracks 1–4*, incubation with 180 nM oligohistidine endonuclease VII; *tracks 5–8*, incubation with 1  $\mu\text{M}$  protein A-endonuclease VII D40N; *tracks 9–12*, incubation with 1  $\mu\text{M}$  protein A-endonuclease VII D40A; *tracks 13–16*, incubation with 60 nM protein A-endonuclease VII D42N; *tracks 17–20*, incubation with 60 nM protein A-endonuclease VII D42A. *Tracks 1, 5, 9, 13, and 17*, junction 3  $5'$ - $^{32}\text{P}$ -labeled on the b strand; *tracks 2, 6, 10, 14, and 18*, junction 3  $5'$ - $^{32}\text{P}$ -labeled on the h strand; *tracks 3, 7, 11, 15, and 19*, junction 3  $5'$ - $^{32}\text{P}$ -labeled on the r strand; *tracks 4, 8, 12, 16, and 20*, junction 3  $5'$ - $^{32}\text{P}$ -labeled on the x strand.

Wild-type sequence endonuclease VII was also expressed as a fusion with an N-terminal oligohistidine sequence by transferring the gene into the plasmid pET-19b. The protein was purified by affinity chromatography on a column to which nickel ions were chelated.

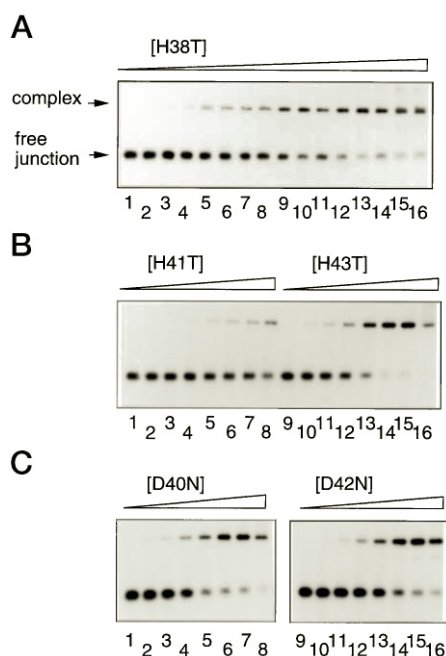
The purity of the proteins was analyzed by polyacrylamide gel electrophoresis in a buffer containing SDS, and the preparations were generally found to contain a single polypeptide migrating at the position expected for the calculated mass (Fig. 2).

The single-amino acid changes introduced into endonuclease VII are summarized in Table I. In general we have altered histidine residues to threonine, glutamine, or serine, and aspartate residues to asparagine or alanine.

**Mutants with Altered Sequences in the His-Acid Cluster Have Normal Zinc Content**—The His-acid cluster is centrally located within the zinc-binding region of endonuclease VII. While previous studies have strongly implicated the four cysteine resi-

dues in the coordination of the zinc ion (23), we could not exclude some role for other amino acids, particularly the histidine residues. We therefore measured the zinc content of mutants representative of each position (as N-terminal protein A fusions) using a colorimetric assay (38). The results are summarized in Table I, where it can be seen that each of the mutant proteins analyzed contains 1 mol of zinc/mol of protein within the probable experimental error. Thus, the zinc content of the protein has not been altered by mutation of any of these residues from the wild-type sequence, strongly indicating the lack of a role in zinc coordination for these amino acids.

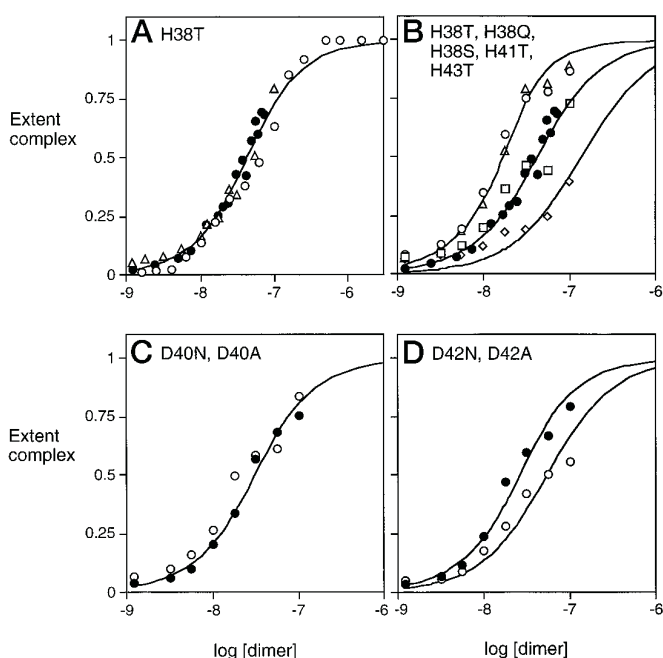
**Activity of Mutant Proteins in the Cleavage of Four-way DNA Junctions**—Wild-type sequence endonuclease VII cleaves four-way DNA junctions with considerable selectivity. It cleaves junctions with the central sequence of junction 3 on two diametrically related strands (called b and r), three bases from the point of strand exchange. The activities of the mutant proteins (as N-terminal protein A fusions) were examined under stand-



**FIG. 4. Binding of endonuclease VII and derived mutant proteins to a four-way DNA junction.** 24.2 nM of radioactively 5'-<sup>32</sup>P-labeled (h strand) junction 3 was incubated with increasing concentrations of endonuclease VII-derived mutant proteins as N-terminal protein A fusions for 10 min at room temperature. Free junction and DNA-protein complexes were separated by electrophoresis in polyacrylamide and visualized by autoradiography. The protein-junction complex migrates as a retarded species relative to the free junction (both species indicated on left in A). *A*, binding of protein A-endonuclease VII H38T to junction 3. *Tracks 1–16*, protein concentrations (calculated for dimeric species) of 1.2, 2.4, 4.8, 7.3, 12.1, 17, 19.4, 24.2, 30.3, 36.4, 42.4, 48.5, 54.5, 60.6, 66.6, and 72.7 nM, respectively. *B*, binding of protein A-endonuclease VII H41T and H43T to junction 3. *Tracks 1–8*, protein A-endonuclease VII H41T concentrations (calculated for a dimeric species) of 1.2, 3.2, 5.6, 10, 17.8, 31.7, 56.2, and 100 nM respectively; *tracks 9–16*, protein A-endonuclease VII H43T concentrations (calculated for dimeric species) of 1.2, 3.2, 5.6, 10, 17.8, 31.3, 56.2, and 100 nM, respectively. *C*, binding of protein A-endonuclease VII D40N and D42N to junction 3. *Tracks 1–8*, protein A-endonuclease VII D40N concentrations (calculated for dimeric species) of 1.2, 3.2, 5.6, 10, 17.8, 31.7, 56.2, and 100 nM, respectively. *Tracks 9–16*, protein A-endonuclease VII D42N concentrations (calculated for dimeric species) of 1.2, 3.2, 5.6, 10, 17.8, 31.7, 56.2, and 100 nM, respectively.

ard conditions, using a four-way DNA junction with the central sequence of junction 3 of Duckett *et al.* (39) (Fig. 3), and the results are summarized in Table I. It is clear that aspartate 40 and histidine 41 are essential to activity, because all mutations of these residues result in total loss of detectable activity (note that we have used a 17-fold higher concentration of the inactive mutant proteins (1  $\mu$ M), compared with the active ones (60 nM)). Histidine 43 is also important, because alteration to threonine leads to an almost total loss of activity. By contrast, aspartate 42 can be replaced by asparagine or alanine without detectable loss of activity. Histidine 38 can be changed to threonine with retention of activity, but H38T has an interesting thermal sensitivity that we shall discuss in a further publication. We have previously reported that endonuclease VII H41T was active (23), but we now believe that this was an error. All subsequent preparations have been totally inactive, and comparison of properties suggests that the mutant proteins H41T and H38T might have been temporarily exchanged at that time. All mutants have now been reconfirmed by sequencing the genes used for their expression.

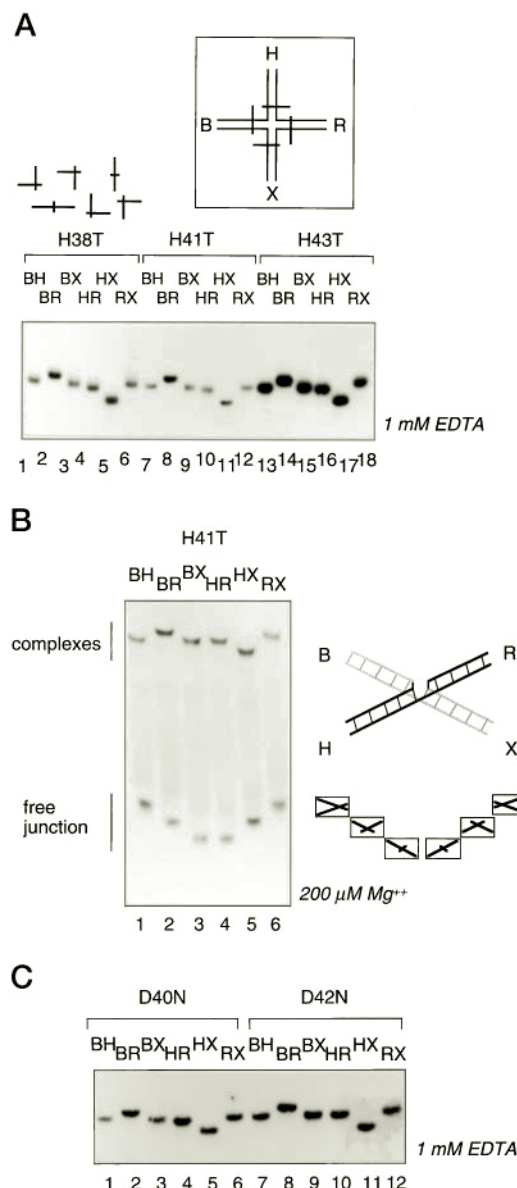
**Binding to Four-way DNA Junctions**—Endonuclease VII of wild-type sequence binds selectively to four-way DNA junctions. This is also true of a non-catalytic mutant protein endo-



**FIG. 5. Binding isotherms for endonuclease VII and derived mutant proteins binding to a four-way DNA junction.** Extent of protein binding to four-way junctions as a function of total protein concentration was estimated by gel electrophoresis (see the legend to Fig. 4). The fraction of DNA junction bound to protein was calculated for each protein concentration and plotted against the protein molarity (calculated for a dimeric species) on a logarithmic scale. The data were fitted to a model for the binding process (see under "Materials and Methods") from which the binding affinities were calculated. The points plotted are experimental data, and the lines are simulations derived using the association constants derived from the fits. *A*, binding of protein A-endonuclease VII H38T. Three independently measured sets of data are plotted, differentiated by the use of three different plotting symbols. The line was calculated for a  $K_a = 3.29 \times 10^7 \text{ M}^{-1}$ . *B*, binding of different protein A-endonuclease VII histidine mutants. Data are shown for the mutant sequences H38T (●), H38Q (□), H38S (○), H41T (◇), and H43T (△). The lines were calculated for  $K_a = 2.0 \times 10^8 \text{ M}^{-1}$ ,  $3.29 \times 10^7 \text{ M}^{-1}$ , and  $7.93 \times 10^6 \text{ M}^{-1}$ . *C*, binding of protein A-endonuclease VII aspartate 40 mutants. Data are shown for the mutant sequences D40N (●) and D40A (○). The line was calculated for a  $K_a = 5.4 \times 10^7 \text{ M}^{-1}$ . *D*, binding of protein A-endonuclease VII aspartate 42 mutants. Data are shown for the mutant sequences D42N (●) and D42A (○). The lines were calculated for  $K_a = 7.0 \times 10^7 \text{ M}^{-1}$  and  $2.35 \times 10^7 \text{ M}^{-1}$ .

nuclease VII E86A; this protein (as either N-terminal fusions or non-fusion) binds to four-way junctions in the presence or absence of magnesium ions and is not displaced by a 1000-fold excess of duplex competitor of the same sequence (27). We examined the binding of the proteins that were mutated in the His-acid cluster to radioactively labeled four-way DNA junction. All were found to bind DNA junctions. Binding titrations were carried out using gel electrophoretic retardation in the presence of 1 mM EDTA (Fig. 4). The titrations are well behaved for all the proteins, giving increasing fractions of a single retarded species as the protein concentration is raised. At protein concentrations higher than 100 nM some super-retarded species could be found in some cases, and thus such data were not used in the calculation of binding affinities.

The ratios of bound and free junction were quantified by phosphorimaging, from which apparent dissociation constants ( $K_D$ ) were calculated assuming binding of a dimeric species (Fig. 5). Most of the proteins bound with affinities that were close to that of the wild-type sequence ( $K_D$  in the range 20–40 nM). Protein A-endonuclease VII H38S and H43T had higher affinity ( $K_D \approx 5 \text{ nM}$ ), while H41T had lower affinity ( $K_D = 96 \text{ nM}$ ). These results indicate that the loss in activity of the proteins with sequence alterations at Asp-40, His-41, and



**FIG. 6. Comparative gel electrophoretic analysis of the global conformation of four-way DNA junction bound to endonuclease VII variants.** Radioactively 5'-<sup>32</sup>P-labeled junction 3 was assembled from four oligonucleotides of appropriate lengths to generate the six species with two long and two short arms that were purified by gel electrophoresis. The four arms of the junction are labeled B, H, R, and X, and the species with shortened arms are labeled by the names of the two long arms. These six species were each incubated with protein A-endonuclease VII variants and analyzed by electrophoresis in a 6% polyacrylamide gel and by autoradiography. **A**, complexes of junction 3 with protein A-endonuclease VII H38T, H41T, and H43T in the absence of added metal ions. The six two-long, two-short arm species generated from junction 3 were electrophoresed in the presence of 1 mM EDTA following incubation with protein. Free junction is not shown in this autoradiograph; the bands arise from the protein-bound junction only. Under these conditions, free DNA junction would generate a slow-fast-slow-slow-fast-slow pattern indicative of the extended square structure with no coaxial stacking of arms (39). Each of the proteins generates the pattern demonstrated previously for protein A-endonuclease VII E86A and H38T (27). This pattern is different from the free DNA and indicates a protein-induced folding of the four-way junction. The resulting global structure is clearly the same for all three histidine mutants. The following double restriction digests were electrophoresed: *tracks 1, 7, and 13*, species BH, with long B and H arms; *tracks 2, 8, and 14*, species BR, with long B and R arms; *tracks 3, 9, and 15*, species BX, with long B and X arms; *tracks 4, 10, and 16*, species HR, with long H and R arms; *tracks 5, 11, and 17*, species HX, with long H and X arms; *tracks 6, 12, and 18*, species RX, with long R and X arms. **B**, complex of junction 3 with protein A-endonuclease VII H41T in the presence of added mag-

His-43 is not due to impairment in substrate binding, since the mutant proteins D40N, D40A, H38T, and H38Q bind normally, and H43T has a 3-fold higher affinity than the enzyme of wild-type sequence.

The binding affinities of inactive mutant proteins could also be measured in the presence of magnesium ions. We found that protein A-endonuclease VII H41T bound around 2-fold more tightly in the presence of 200 μM magnesium ions. The binding affinity of protein A-endonuclease VII D40N was increased by a factor of 1.3 under the same conditions.

**Distortion of the Global Structure of Junctions on Binding Endonuclease VII Variants**—On binding to junctions, endonuclease VII induces a change in the global configuration of arms, demonstrated by comparative gel electrophoresis studies (27). In this method a four-way junction with arms of 40 bp each in length is subjected to shortening of two arms by restriction cleavage in the six possible combinations (39–41). The electrophoretic mobility in polyacrylamide of these six two-long, two-short arm species are compared and analyzed on the basis of the expected relationship (42) between electrophoretic mobility and the angle included between the two long arms. We used this method originally to analyze the structure of the free junction under different conditions (39), but it has more recently been applied to junction-protein complexes (22, 27, 43).

Both endonuclease VII H38T and E86A induce a change in the global folding of DNA four-way junctions (27). The same structure is generated by the inactive mutant endonuclease VII E86A in either the presence or absence of added magnesium ions and is different from that of the free DNA junction under either set of conditions. It is clear that the four-way junction is extensively manipulated by endonuclease VII, and we asked whether the mutant proteins retained the ability to induce the same structural alteration.

The binding of protein A-endonuclease VII to junction 3 generates a pattern of electrophoretic mobilities described by intermediate-slow-intermediate-intermediate-fast-intermediate (27). The relative mobility of the BR species (second from left, as our gels are conventionally loaded) is slower when the protein binds as a protein A fusion. The pattern is interpreted

nesium ions. Since this mutant is catalytically inactive it can be studied in the presence of magnesium ions. The six two-long, two-short arm species were incubated with protein A-endonuclease VII H41T in the presence of 200 μM magnesium ions. The products were analyzed by electrophoresis in 6% polyacrylamide containing 100 μM magnesium ions and by autoradiography. For each of the long-short arm species, two radioactive bands are evident, corresponding to free DNA and complex. The free DNA exhibits the slow-intermediate-fast-fast-intermediate-slow pattern indicative of the stacked X structure with B on X coaxial stacking indicated at the right (39). The structure of this stacking isomer gives rise to the three pairs of long-short arm species in which the included angles between the long arms are acute (BH and RX are slow species), obtuse (BR and HX are intermediate species), or linear (BX and HR are fast species). This interpretation is summarized below the schematic of the stacked X structure on the right. The pattern of mobilities for the long-short species of the junction-protein complex is clearly different from that of the free DNA and is the same as that found in the presence of EDTA (compare with A). The electrophoretic pattern (and thus the global structure of the DNA junction in the presence of this mutant) is unchanged by the presence or absence of magnesium ions, just as was found previously for the complex with protein A-endonuclease VII E86A. *Track 1*, BH species; *track 2*, BR species; *track 3*, BX species; *track 4*, HR species; *track 5*, HX species; *track 6*, RX species. **C**, complexes of junction 3 with protein A-endonuclease VII D40N and D42N in the absence of added metal ions. Free junction is not shown in this autoradiograph. Once again, both proteins generate the same pattern of electrophoretic mobilities found for the other proteins. Thus, all the variants of endonuclease VII appear to impose the same global structure on the four-way DNA junction. *Tracks 1 and 7*, BH species; *tracks 2 and 8*, BR species; *tracks 3 and 9*, BX species; *tracks 4 and 10*, HR species; *tracks 5 and 11*, HX species; *tracks 6 and 12*, RX species.



in terms of a principal binding across arms H and X (in which the cleavages are introduced by the active enzyme in the presence of magnesium ions) and a rotation of arms B and R toward arms H and X, respectively, together with a movement out of the plane (27).

Fig. 6A compares the electrophoretic patterns of the complexes of junction 3 with protein A fusions of the three histidine-to-threonine mutants of endonuclease VII, in the presence of 1 mM EDTA (TBE buffer) to prevent cleavage by active enzyme. The patterns of mobilities of the six long-short species are identical for all three proteins, indicating that all three induce the same global conformation of arms on the four-way DNA junction. The experiment was repeated for protein A endonuclease VII H41T in the presence of 200  $\mu$ M magnesium ions (TBM buffer), conditions where the free DNA junction folds into the stacked X structure. Since this mutant is completely inactive as a nuclease, the experiment can be carried out in the presence of magnesium ions without inducing cleavage of the DNA. Despite the change of conditions, the complex clearly has the same global structure, resulting in an unchanged pattern of electrophoretic mobilities (Fig. 6B).

The aspartate mutants also generated the same structure in the four-way junction. Fig. 6C shows the comparative gel electrophoretic analysis of the complexes of the six long-short variants of junction 3 with endonuclease VII D40N and D42N in the presence of 1 mM EDTA (TBE buffer). Once again the pattern is unchanged from those of all the endonuclease VII variants.

These results suggest that the binding process is very similar for all of the mutants of the His-acid cluster studied. This is further evidence that the lack of catalytic activity in some mutant proteins was not due to impairment of binding.

#### DISCUSSION

The mutation analysis confirms that amino acids contained within the conserved His-acid cluster are important in the function of T4 endonuclease VII. In particular, one aspartate (Asp-40) and two histidine residues (His-41 and His-43) are required for cleavage of DNA junctions. Since mutation of these residues leads to only small changes in binding affinity for DNA junctions, it is likely that they are involved (directly or indirectly) in the catalysis of phosphodiester bond hydrolysis. The zinc content of none of the mutants was significantly altered from 1 mol/mol of protein, and thus a role in the coordination of zinc is not likely. We have previously shown (27) that another acidic residue, glutamate 86, is essential for catalytic activity. This is located in the second region of the primary sequence of the protein, which exhibits some similarity to endonuclease I of T7. It is therefore possible that the active site of the enzyme comprises amino acid side chains from both of these regions of the polypeptide. We suspect that acidic side chains may be involved in the coordination of a magnesium ion required for the cleavage reaction. This is a common feature of nucleases (33–35), including the *E. coli* junction-resolving enzyme RuvC (36), where the catalytic center contains three aspartate residues and one glutamate residue.

All the endonuclease VII sequence variants examined retained selective binding to DNA junctions. This is a further indication of the divisibility of binding and catalysis in this enzyme. Small changes in binding affinities were measured over the set of mutant proteins, but this difference was only about 20-fold between the tightest (endonuclease VII H38S) and weakest binding (endonuclease VII H41T) protein, corresponding to an overall difference in binding free energy of 1.7 kcal mol<sup>-1</sup>. In most cases the differences are much smaller than this. This suggests that sequence changes in the His-acid cluster tend to affect interactions primarily with the transition

state rather than the ground state DNA structure. It is interesting to note that the mycobacteriophage L5 gp59 protein has a serine at the position corresponding to histidine 38 in endonuclease VII. This protein therefore has the same sequence at this position as the tightest binding endonuclease VII variant.

An interesting feature of the binding of endonuclease VII to four-way junctions is the change in the global structure of the DNA (27). Distortion of DNA structure upon binding of junction-selective proteins appears to be rather general, having also been observed for T7 endonuclease I (22), *E. coli* RuvA (43) and RuvC (26), and yeast CCE1.<sup>2</sup> The distortion imposed on the global configuration of helical arms in the junction by endonuclease VII is quite significant, and we have proposed a model of the structure of the bound junction involving an unstacking of the arms at the point of strand exchange (27). All of the sequence variants studied here appear to induce the same change in junction structure, whether active or inactive, despite small differences in binding affinity. This further supports the contention that the basic binding processes are unaltered by any of the sequence changes in the His-acid cluster. This is also indicated by the fact that mutations within the region result in either proteins that cleave at exactly the same positions as the wild-type enzyme or fail to cleave at all. None causes an alteration in the cleavage pattern that might be expected if the manner of substrate binding had been changed.

In summary, we have found that a number of sequence changes in the His-acid cluster of endonuclease VII lead to reduced activity of the enzyme. In particular aspartate 40 and histidines 41 and 43 appear to be required for cleavage of DNA junctions. However, none of these mutations appears to affect binding to DNA junctions (beyond relatively small changes in affinity) or the distortion of DNA structure. It is therefore quite likely that the His-acid cluster will prove to be important in generating the active site of T4 endonuclease VII.

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